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GLUTAMATE DIFFERENTIALLY INHIBITS THE EXPRESSION OF CLASS II MHC ANTIGENS ON ASTROCYTES AND MICROGLIA[1]

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MHC molecules are required for Ag recognition by T cells. Inasmuch as cells in the central nervous system do not express MHC constitutively, appearance of MHC, in inflammatory and degenerative diseases of the brain, may indicate local Ag presentation and subsequent immune response. Although both astrocytes and microglia are capable of class II MHC expression in vitro, in vivo studies failed to show the presence of significant amounts of class II on astrocytes compared to microglia. Our study is designed to clarify possible regulatory mechanisms that can explain the differences in inducibility of class II MHC between astrocytes and microglia in vivo. Using dissociated rat brain cell cultures, we have found that glutamate, an excitatory neurotransmitter, exerted a profound inhibitory effect on IFN-γ-induced expression of class II on astrocytes, but not on microglia. Both glutamate and norepinephrine, a neurotransmitter previously reported to down-regulate class II on astrocytes, inhibited the induction of class II on astrocytes by eliminating accumulation of class II MHC mRNA. The kinetics of class II mRNA induction by IFN-γ in the presence of glutamate suggested that glutamate may act as a transcriptional inhibitor. It is likely that class II induction on astrocytes in vivo may be selectively down-regulated by neurotransmitters such as glutamate and norepinephrine.

Expression of MHC molecules determines the ultimate responsiveness of humoral and cellular immunity by stimulating T cell subsets in an Ag-specific, MHC-restricted manner. Although class I MHC is widely expressed, class II expression is restricted to certain cells such as B cells, dendritic cells, and macrophages (1, 2). In the CNS[4], glial cells, which include astrocytes, oligodendrocytes, and microglia, the resident macrophages derived from bone marrow, express neither class I nor class II molecules (3, 4). However, glial cells in vitro spontaneously express class I molecules, and astrocytes and microglia can be induced to express class II molecules (5–7). Primary astrocytes from Lewis rats, a strain susceptible to EAE, express higher levels of class II molecules when stimulated with low doses of IFN-γ when compared to astrocytes from the EAE-resistant Brown Norway rats (8), whereas class II inducibility on macrophages and microglia from these two strains appears similar (8–10). In CNS tissues from animals with EAE, graft-vs-host disease (11, 12), and from patients with active multiple sclerosis (13, 14), class II-bearing astrocytes are rare in contrast to the appearance of numerous class II$^+$ microglia. In addition, systemic infusion of IFN-γ selectively induced class II on microglia but not on astrocytes (15), although its in vitro induction is similar for both cell types. These observations collectively indicate that regulation of class II expression on astrocytes may differ from that of macrophages and microglia, and the class II expression on astrocytes but not microglia may be actively down-regulated in the CNS. Inhibition of class II expression induced by IFN-γ on astrocytes by norepinephrine shown by Frohman et al. (16) also supports such speculation.

To investigate possible mechanisms that may explain the differential in vivo expression of class II by astrocytes and microglia, we have compared class II expression on rat astrocytes and microglia in the presence of various neurotransmitters. Glutamate, an excitatory amino acid abundantly present in the CNS (17), and norepinephrine were able to inhibit the surface expression of class II proteins and the accumulation of class II mRNA in astrocytes but not in microglia.

MATERIALS AND METHODS

Chemicals and reagents. Rat rIFN-γ was from Amgen (Thousand Oaks, CA). LPS (*Escherichia coli* 0127:B8), L-glutamate (free acid), monosodium glutamate, norepinephrine, adenosine, serotonin, acetylcholine, glycine and forskolin were from Sigma Chemical Co. (St. Louis, MO). H-7 and HA-1004 were from Seikagaku (Rockville, MD). Mouse mAb to rat polymorphic Ia (MRC OX-6), class I MHC and Mac-1 (MRC OX-42, CD11b, or iC3b receptor) were from Bioproducts for Science (Indianapolis, IN). FITC-conjugated goat anti-mouse IgG1 and IgG2a, as well as normal mouse IgG1 and IgG2a were from Southern Biotechnology (Birmingham, AL).

Glial cell culture. Dissociated cell cultures were prepared from Lewis or Sprague-Dawley rat brains as described previously (18). Briefly, brains were removed from 1- to 2-day-old rats. After removal of meninges, cerebral tissues were mechanically dissociated in DMEM/F12 and passed sequentially through 210- and 130-μm size nylon meshes. Cells were suspended in DMEM/F12 containing 10% heat-inactivated FCS and seeded into 75-cm^2 flasks (Corning, Corning, NY) at a density of 7.5×10^5 cells/ml. Typically, astrocytes reached confluency approximately 8 to 10 days after seeding, and were overlayered by oligodendrocytes and microglia. The flasks were shaken at 250 rpm for 1 h and the media, rich in microglia, were placed in 10-cm plastic petri dishes (Falcon Labware, Oxnard, CA). After incubation for 1 h at 37°C, dishes were vigorously washed to

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[4] Abbreviations used used in this paper: CNS, central nervous system; EAE, experimental allergic encephalitis; PKC, protein kinase C; DMEM/F12, DMEM/Ham's/f12 containing 100 U/ml penicillin and 100 μg/ml streptomycin; XBP, X-binding protein.

remove nonadherent cells. Experiments with microglia were performed on the same day of isolation. For astrocytes, culture flasks were further shaken at 250 rpm for 24 h followed by 130 rpm for 48 h (19) to remove oligodendrocytes and the residual microglia. The astrocytes remaining adherent were subcultured at 3×10^5 cells/well in a 24-well plate (Corning) after trypsinization. Cells were grown for another 2 to 3 days to reach confluency.

Induction of class II MHC proteins and FACS analysis. Stock solutions of neurotransmitters were made in PBS and further diluted in DMEM/F12 before use. Ascorbic acid (1 mg/ml) was added to norepinephrine stock solution to prevent oxidation. Cells were stimulated with IFN-γ or LPS in the presence or absence of neurotransmitters for 48 h for the mRNA analysis and 72 h for FACS to detect class II protein expression.

After stimulation, astrocyte monolayers were washed in Ca^{2+} and Mg^{2+}-free HBSS, and dissociated using 0.25% trypsin/0.02% EDTA in HBSS. Microglia were scraped 5 min after the addition of trypsin solution to achieve maximum cell yield. To ensure that class II Ag are trypsin resistant, some monolayers were collected by scraping only; no apparent differences were noted in the degree of IFN-γ-induced class II expression. HBSS containing 5% FCS and 0.2% sodium azide was used to dilute antibodies and for cell washing. Cells were incubated for 1 h at 25°C with anti-class II IgG (1/100 dilution), then for 30 min with FITC-conjugated goat IgG to mouse IgG (1/50 dilution). For microglia, 10% normal goat serum was added to the antibody solutions to prevent the antibody binding through the FcR. After a final washing, cells were suspended in 2% paraformaldehyde in HBSS and subjected to FACS analysis (Becton Dickinson, Mountain View, CA). The channels were gated to exclude dead cells and debris and samples with the appropriate control isotype antibody were used to give approximately 1% background. Analysis of 10,000 cells from each sample was recorded. Class II expression on unstimulated astrocytes and microglia were less than 2%.

Northern blot analysis. Astrocytes in 75-cm^2 flasks were stimulated with IFN-γ with or without glutamate, norepinephrine, forskolin, H-7, or HA-1004 for the indicated time period. Total cellular RNA was extracted by the guanidinium isothiocyanate method and purified by ultracentrifugation through a cesium chloride cushion (20). A total of 10 to 20 μg of formaldehyde-denatured RNA was loaded in each lane and electrophoresed through 0.8% agarose gel. The RNA was transferred to nitrocellulose paper, and hybridized with ^{32}P labeled probes using random primer oligolabeling kit from Pharmacia Fine Chemicals, Piscataway, NJ. The probes consisted of 1.3-kb *Hin*dIII 3′genomic fragment of mouse I-A α^b gene subcloned in pGEM4 and 960-bp mouse XBP cDNA in pBS KSII (gifts of Dr. L. Glimcher, Harvard University, Cambridge, MA). Hybridization was carried for 2 days at 37°C. The blots were washed twice in 2xSSC/0.1% SDS (1xSSC/0.15 M NaCl/0.15 M sodium citrate) and twice in 0.5xSSC/0.1% SDS at 55°C, and exposed to Kodak OMAT AR5 film using intensifying screen at -70°C, for 3 to 5 days. The same blots were stripped and hybridized for mRNA encoding β-actin as control. The mRNA were quantitated by measuring OD of the corresponding bands on the autoradiograph using the Computing Densitometer (Molecular Dynamics, Sunnyvale, CA). The integrated volume of each band was calculated using the Imagequant software (Molecular Dynamics).

RESULTS

Immunophenotypes of glial cells in culture and induction of class II proteins. Microglia cultures examined at the end of 72 h incubation showed more than 90 to 95% cells stained for Mac-1, whereas less than 2% cells were positive in astrocyte cultures (Fig. 1*A*, *a* and *c*). About 97% of cells in microglia cultures were positive for IgG FcR (data not shown). Stimulation with 50 U/ml IFN-γ effectively induced class II expression (Ia) on microglia (Fig. 1*A*, *b*) and astrocytes (Fig. 1*A*, *d*). Although microglia were more responsive than astrocytes, IFN-γ induced class II expression in a dose-dependent manner on both cell types (Fig. 1*B*).

Glutamate inhibited IFN-γ-induced class II on astrocytes, but not on microglia. To identify the inhibitors of class II induction, we incubated cells with IFN-γ in the presence or absence of various neurotransmitters (Table I). Glutamate and norepinephrine showed an inhibitory effect on astrocytes. Glutamate was capable of inhibiting

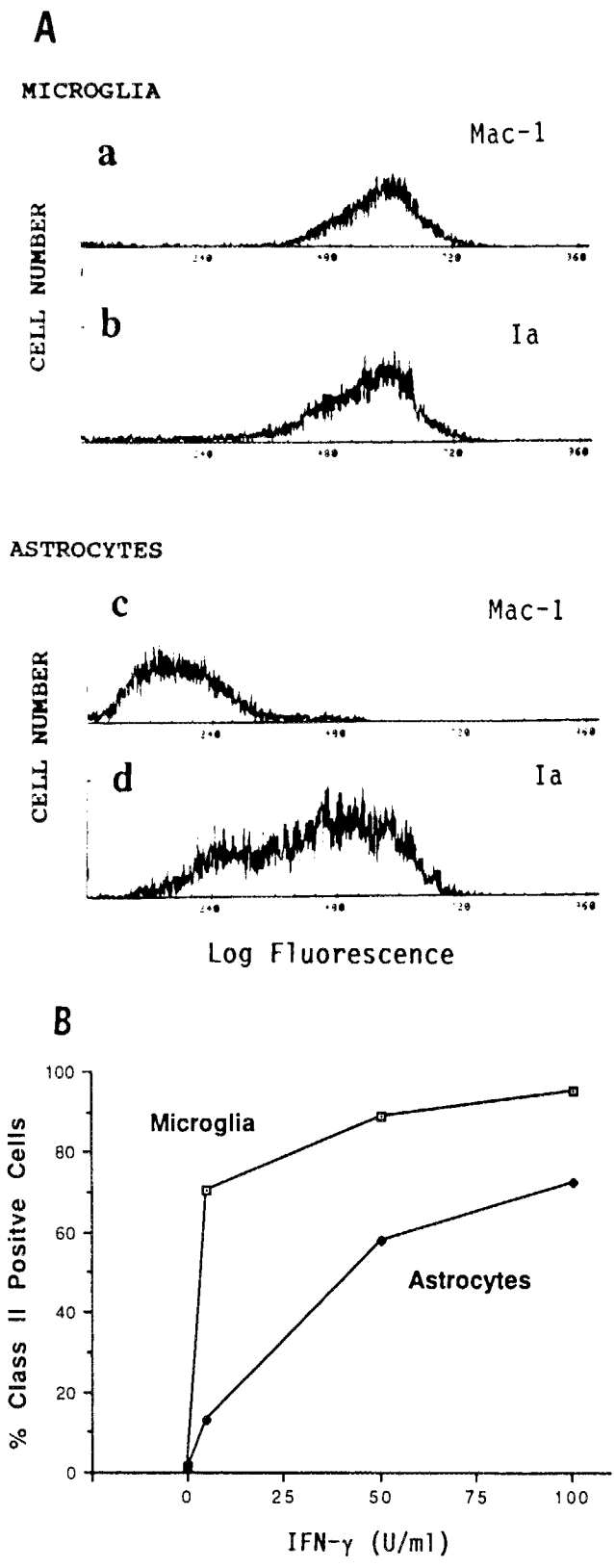


Figure 1. *A*, Phenotypic characterization of microglia and astrocytes in cultures. The surface expression of Mac-1 and class II molecules were assessed by indirect immunofluorescence staining and flow cytometry as described. More than 95% cells in microglial cultures expressed surface Mac-1 (*a*), whereas 2 to 3% stained for Mac-1 in astrocyte cultures (*c*). Both cells, microglia (*b*) and astrocytes (*d*), expressed class II molecules (Ia-Ag) when incubated with IFN-γ (50 U/ml) for 72 h. *B*, Astrocytes (◆) and microglia (□) treated with varying doses of rat rIFN-γ for 72 h were examined for cell-surface class II molecules by FACS. Results are representative of more than seven separate experiments.

TABLE I

Effect of neurotransmitters on IFN-γ-induced class II expression in astrocytes[a]

Neurotransmitter	Dose (M)	Percent Inhibition[b]
Glutamate	10^{-3}	73
Norepinephrine	10^{-3}	75
Acetylcholine	10^{-3}	0
GABA	10^{-3}	0
Glycine	10^{-3}	0
Adenosine	10^{-4}–10^{-6}	0
Serotonin	10^{-4}–10^{-6}	0

[a] Astrocyte monolayers stimulated for 72 h with IFN-γ (5 U/ml) in the presence or absence of neurotransmitters (NT) were assessed for the cell-surface class II expression as described in *Materials and Methods* and in the Legend of Figure 1. Five U/ml of IFN-γ-induced class II MHC on 30% of astrocytes.

[b] Percent inhibition

$$= \frac{\%\ \text{class II}^+\ \text{cells (IFN)} - \%\ \text{class II}^+\ \text{cells(IFN + NT)}}{\%\ \text{class II}^+\ \text{cells (IFN)}} \times 100.$$

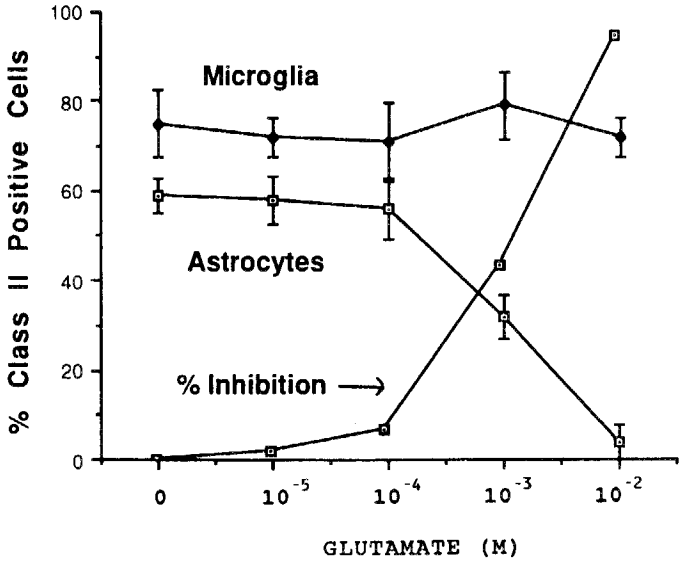


Figure 2. Glutamate inhibited class II MHC expression on astrocytes but not on microglia. Astrocytes (□) and microglia (◆) stimulated with 50 U/ml IFN-γ with or without glutamate (10^{-2} M) for 72 h were analyzed for class II expression by FACS. Percent inhibition was calculated as 100 × ((% positive cells with IFN-γ- % positive cells with IFN-γ and glutamate) divided by % positive cells with IFN-γ). Numbers are expressed as mean ± SD from more than six experiments for astrocytes and two experiments for microglia performed in duplicates.

class II expression on astrocytes with 95% inhibition achieved at a concentration of 10^{-2} M as shown in Figure 2. The normal concentration of glutamate in whole brain homogenates is reported to be 10^{-2} M (17, 21). In contrast to astrocytes, glutamate failed to inhibit class II expression on microglia (Fig. 2). The effect of norepinephrine on class II expression in astrocytes and microglia is shown in Figure 3. Norepinephrine, at a 10^{-4} M concentration, produced approximately 25% inhibition in astrocytes, but showed no effect on microglia in multiple experiments. In microglia 10^{-3} M norepinephrine was also ineffective (results not shown) whereas the 10^{-2} M showed oxidation of the microglia culture medium despite the addition of ascorbic acid.

Glutamate and norepinephrine prevent class II mRNA accumulation in astrocytes. The possible inhibitory sites of glutamate and norepinephrine were explored by Northern analysis using mouse I-A probe. Figure 4*A* demonstrates a dose-dependent decrease in class II mRNA accumulation at doses consistent with those required to inhibit cell surface class II expression. Norepi-

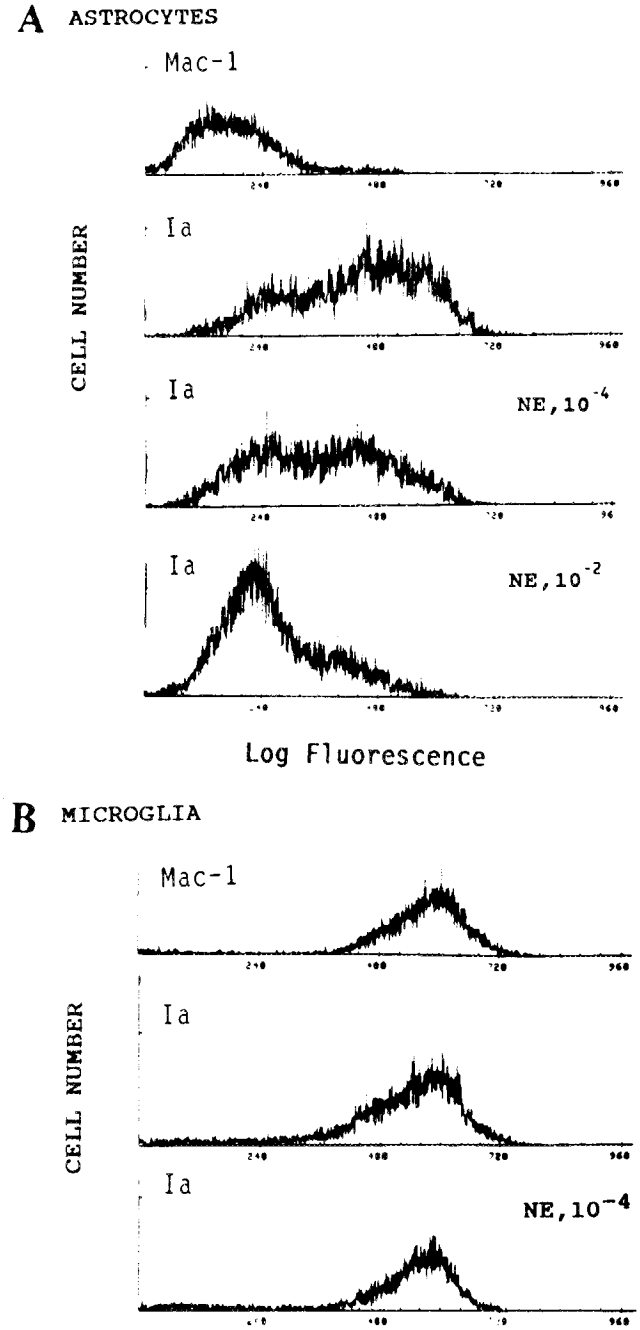


Figure 3. Effects of norepinephrine on IFN-γ-induced class II expression. Astrocytes (*A*) and microglia (*B*) were stimulated with 50 U/ml IFN-γ for 72 h with or without norepinephrine. Microglia showing Mac-1 staining are shown in the *upper panel*. Norepinephrine, at 10^{-4} and 10^{-2} M, produced 35 and 85% inhibition, respectively in astrocytes (*A*), whereas no inhibition was noted at 10^{-4} M norepinephrine in microglia.

nephrine also inhibited class II mRNA expression (Fig 4*B*). The kinetics of class II mRNA accumulation in IFN-γ-stimulated astrocytes with and without 10^{-2} M glutamate, are shown in Figure 5. The class II mRNA appeared around 12 h, and increased steadily up to 72 h. Glutamate inhibited the class II mRNA accumulation at all time points although the fold inhibition varied. The inhibition increased from 3.7-fold at 12 h to 4.5- and 8.4-fold at 24 and 36 h, respectively. Beyond 36 h the level of inhibition decreased to fourfold at 48 h and threefold at 72 h. The mRNA encoding XBP also increases with time in cells stimulated with IFN-γ, although this XBP increase was not affected by glutamate (Fig. 5*B*).

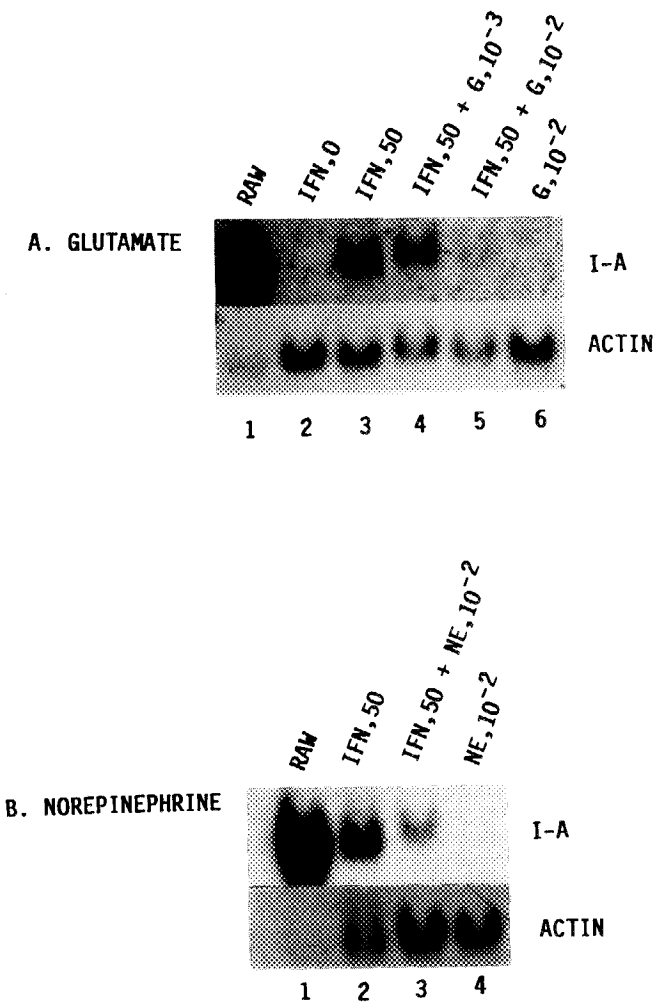


Figure 4. Glutamate (*A*) and norepinephrine (*B*) inhibited class II mRNA accumulation in astrocytes induced by IFN-γ. Total RNA (10 μg) from astrocytes treated with medium alone (*lane 2*), 50 U/ml IFN-γ (*lane 3*), IFN-γ with 10^{-3} M (*lane 4*), 10^{-2} M (*lane 5*) glutamate, or glutamate alone (10^{-2} M) (*lane 6*) were hybridized with probes for I-A and also for β-actin. *B*, Astrocytes exposed to 50 U/ml IFN-γ (*lane 2*), IFN plus 10^{-2} M norepinephrine (*lane 3*) or norepinephrine alone (*lane 4*) were also probed for I-A and β-actin. *Lane 1*, RNA (1 μg) from RAW 264.7 cells stimulated with IFN-γ (50 U/ml).

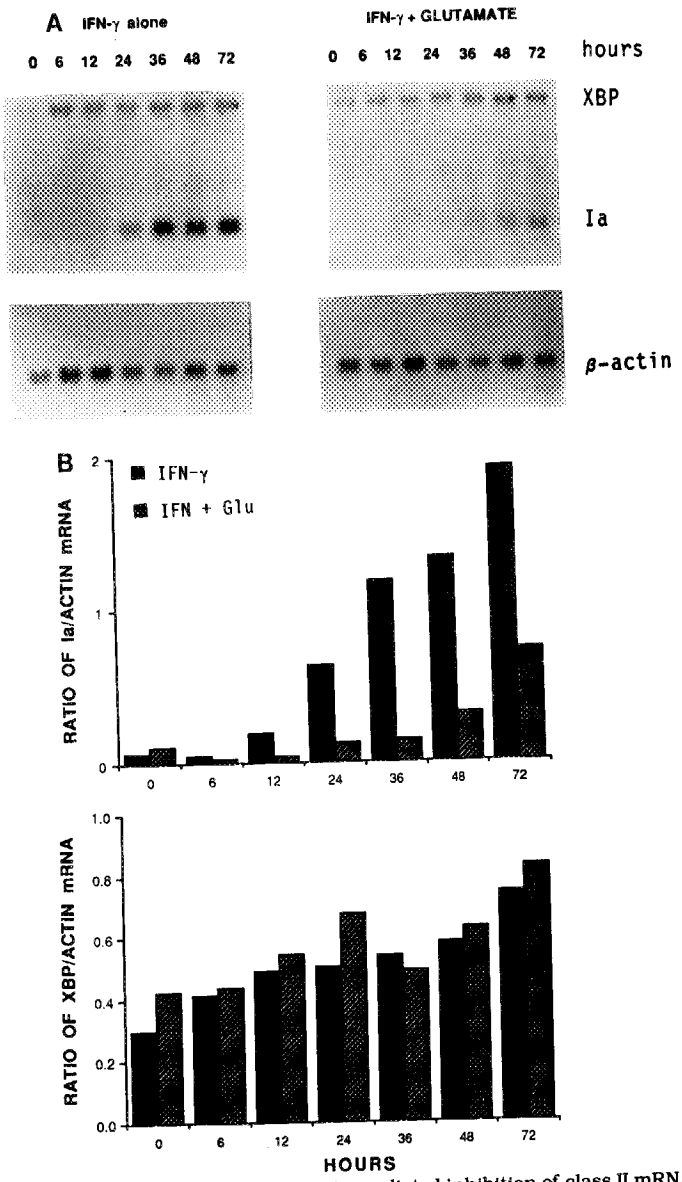


Figure 5. Kinetics of glutamate-mediated inhibition of class II mRNA. *A*, Astrocytes were incubated with IFN-γ (100 U/ml) or IFN-γ plus 10^{-2} M glutamate and RNA isolated at indicated time points were examined by Northern blots for mRNA encoding class II MHC, XBP, and β-actin. *B*, Ratios of the integrated volume of mRNA densities for class II mRNA/β-actin (*upper panel*) and XBP/β-actin (*lower panel*) were obtained by densitometric analysis of the autoradiograph shown in (*A*). IFN-γ alone (■) and IFN-γ plus 10^{-2} M glutamate (▨).

Inhibition by glutamate is not specific for IFN-γ-induced class II MHC. We further tested whether the effect of glutamate is limited to IFN-γ by examining the LPS-induced class II expression. As reported previously (7), LPS can induce class II on a small proportion of astrocytes. We noted a maximum response at 20 ng/ml LPS (Fig. 6). Glutamate, at 10^{-2} M, was able to inhibit LPS-induced class II expression (Fig. 6). However, the effect appeared more significant when higher concentrations of LPS were used. To investigate whether the glutamate effect on astrocytes involves down-regulation of IFN-γ receptors, we examined its effect on IFN-γ-induced changes in class I MHC expression. Over a period of several days, class I expression occurred spontaneously on majority of astrocytes in vitro. However, the fluorescence intensity, which reflects the number of class I molecules per cell, increased with IFN-γ in a dose-dependent manner. Figure 7 demonstrates that glutamate failed to inhibit this effect of IFN-γ on class I MHC expression. Furthermore, addition of glutamate 1, 2, and 3 h before or 1 h after IFN-γ had no effect on the degree of class II inhibition (results not shown). These findings indicate that the action of glutamate was not through IFN-γ receptor down-regulation.

Astrocyte class II induction by IFN-γ is through cAMP and protein kinase C-dependent pathway. Previous reports showed that inhibition of class II expression by norepinephrine occurred through the cAMP pathway. We explored the second messengers involved in the glutamate effect on astrocyte class II regulation. We used forskolin, an inducer of cAMP (22), and H-7 and HA-1004, inhibitors of protein kinases (23). Incubation of cells with forskolin and IFN-γ resulted in a dose-dependent inhibition of class II on astrocytes with a maximum 80% inhibition at 50 μM forskolin (Fig. 8*A*). In addition, complete inhibition of class II expression was achieved with H-7, but not with HA-1004 (Fig. 8*B*) Northern analysis also showed that both Forskolin and H-7 inhibited the expression of class II mRNA by IFN-γ (data not shown). H-7 is a potent inhibitor of PKC and HA-1004,

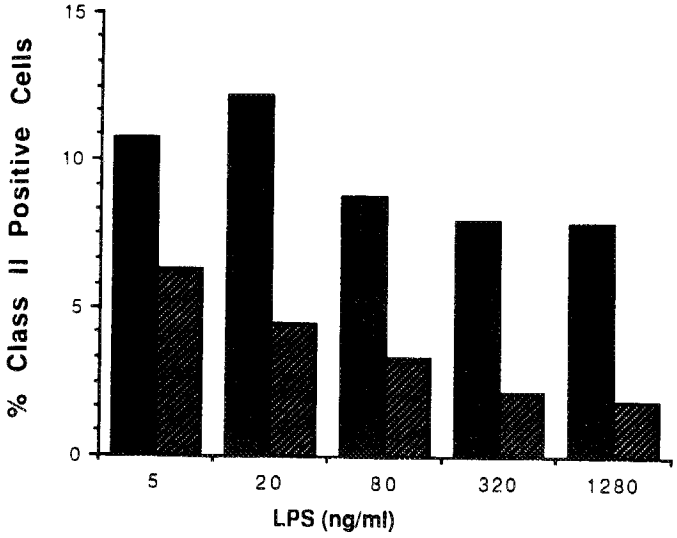


Figure 6. Glutamate inhibited LPS-induced class II on astrocytes. Astrocytes were incubated with LPS alone (■) or LPS plus 10^{-2} M glutamate (▨) for 72 h and class II expression was determined by FACS. The degree of inhibition by glutamate was more significant at higher doses of LPS. A representative result from two separate experiments performed in duplicates is shown as a mean value.

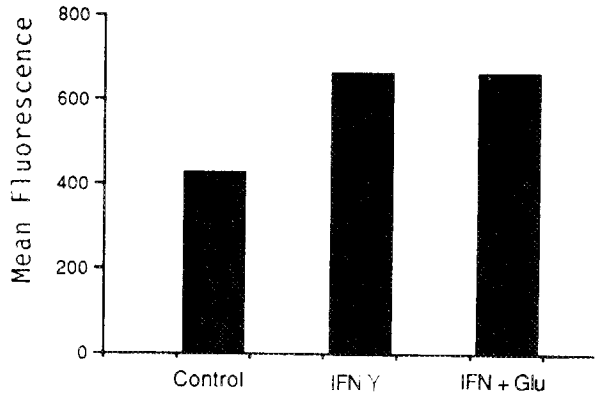
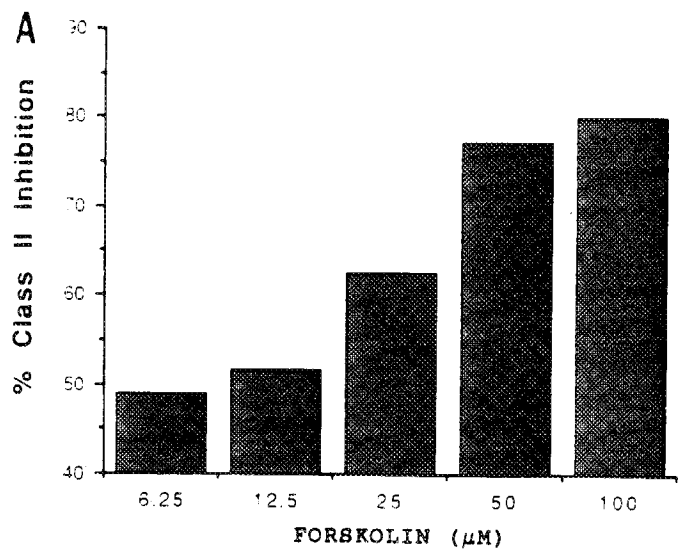


Figure 7. Class I MHC expression enhanced by IFN-γ was not affected by glutamate. Astrocytes were incubated with 5 U/ml IFN-γ with or without 10^{-2} M glutamate for 48 h, then the class I Ag were examined by FACS analysis.



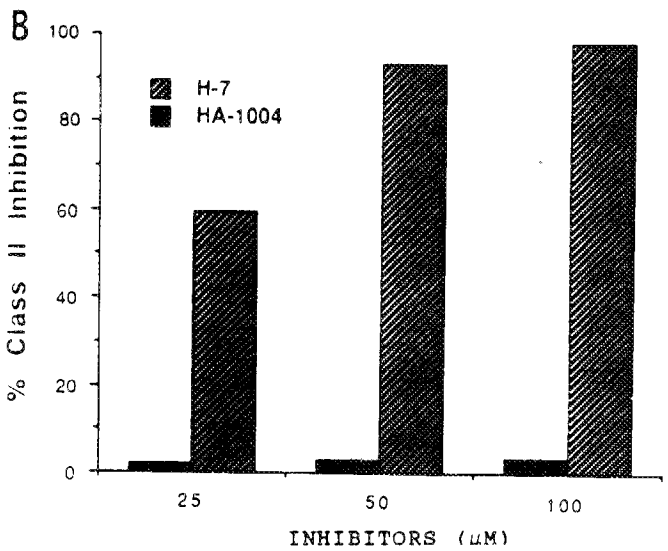


Figure 8. IFN-γ induction of astrocyte class II involves cAMP and PKC-dependent pathways. Astrocytes were incubated with IFN-γ with varying concentrations of forskolin (*A*), H-7 or HA-1004 (*B*) for 72 h before the FACS analysis for class II expression. The % inhibition was calculated using % class II^+ cells achieved by 50 U/ml IFN-γ without inhibitors as 100%.

an H-7 analog commonly used as a control for H-7, is a more selective inhibitor of cGMP and cAMP-dependent kinases with a very weak inhibitory effect on PKC (23). Thus, class II induction in astrocytes by IFN-γ appeared to be PKC dependent as also shown previously (24). cAMP, which was also involved in the down-regulation of class II on astrocytes (16) (Fig. 8*A*) may not act in a kinase-dependent manner.

DISCUSSION

Microglia and astrocytes are the resident CNS cells capable of expressing class II MHC molecules and presenting Ag to T cells in the CNS. Several lines of evidence suggested that regulation of class II MHC in astrocytes may differ from that of microglia. Systemic infusion of IFN-γ in rodents revealed that microglia was the major cell type expressing class II MHC in the brain. Similarly, microglia, not astrocytes, are the frequent target cells in which class II molecules are induced in animals with EAE, graft-vs-host disease, and in patients with multiple sclerosis (11–15). In these studies, class II-bearing astrocytes were infrequently observed.

Our study is designed to identify possible mechanisms that may explain differential expression of class II MHC between astrocytes and microglia in vivo and to identify endogenous mediators of class II down-regulation in astrocytes. Our finding that glutamate, an excitatory neurotransmitter abundantly present in the CNS, exerted profound inhibitory effects on IFN-γ induction of class II on astrocytes, but not on microglial cells, suggests that such a mechanism may be operating in vivo. Furthermore, the down-regulatory effect of glutamate was almost complete at a dose of 10^{-2} M, a reported concentration of glutamate in normal brain homogenate (17, 21). Norepinephrine, previously found to suppress astrocyte class II induction by IFN-γ (16), also failed to suppress microglial class II in our study. Suppression of class II expression with forskolin in astrocytes (Fig. 8*A*) is in agreement with previous studies that have shown that an increase in cAMP in astrocytes in response to norepinephrine occurs through activation of β-adrenergic receptors (25) and this mechanism was proposed to be responsible for the inhi-

bition of class II by norepinephrine (16). The effect of glutamate may also be similar and it is probable that the PKC-dependent pathways required for class II expression may be a target for the glutamate effect. In this context it is interesting that glutamate also inhibited LPS-induced class II on astrocytes because LPS and IFN-γ are both capable of stimulating PKC, as suggested in class II induction on astrocytes (7).

Whether the differential inhibition of class II on glial cells may be simply due to the absence of specific receptors on microglia remains to be determined. Although the existence of glutamate receptors on microglia is unknown, β-adrenergic receptors are known to be present on rat microglia as measured by agonist-stimulated intracellular Ca^{2+} release (K. McCarthy, personal communication). Thus, it is possible that cell type-specific regulatory factors that govern class II expression could be modulated by neurotransmitter-mediated signal transduction.

Expression of class II can be regulated by transcription factors interacting with 5′ upstream elements of the gene that include the highly conserved X and Y box motifs (26). Translation and transport of class II proteins also follow a complex series of post-transcriptional processing (27). First, inhibitory sites of glutamate and norepinephrine on expression of class II proteins appeared to involve the expression of class II mRNA. Both glutamate and norepinephrine abolished the class II mRNA accumulation at doses that inhibited the expression of the cell surface protein on astrocytes. Inasmuch as glutamate reduced class II mRNA expression throughout the experiment, with more potent inhibition at earlier time points, it suggested that glutamate may operate at the level of transcription. Recently, it has been shown that cAMP down-regulates class II gene expression by inhibiting or altering transcription factors that bind to the class II gene promoter elements, S and X1 (28). XBP, a transcription factor known to bind the X2 element of I-Aα promoter, has been implicated in regulating class II expression (29, 30). In astrocytes, XBP expression was enhanced 2.5-fold by IFN-γ at 72 h and glutamate was ineffective in reducing the accumulation of XBP mRNA. Therefore, glutamate may affect other IFN-γ-induced regulatory proteins whose target elements on the class II gene are located outside the X2-box as alluded to by others (31, 32). It appears that the glutamate effect was not due to down-regulation of IFN-γ receptors because IFN-γ mediated class I enhancement was not reduced by glutamate and class II expression was unchanged when glutamate was added either 3 h before or 1 h after IFN-γ. Furthermore, the glutamate effect was not IFN-γ specific because LPS-stimulated class II was also reduced by glutamate.

We have also explored the glutamate receptor subtypes involved in class II down-regulation using receptor agonists, quisqualate, kainate, and *N*-methyl D-aspartate. At concentrations from 10^{-5} to 10^{-3} M of each of these agonists, the class II protein induced by IFN-γ was partially inhibited (data not shown). These preliminary data do not support the coupling of a single receptor subtype, or the existence of a rank order of agonist action in mediating class II down-regulation (33).

The differential regulation of class II in astrocytes and microglia suggest that astrocytes may be under negative regulatory influence in vivo by neurotransmitters such as glutamate and norepinephrine, that can explain the higher incidence of class II^+ microglia in the brain (34). Although a potential role for astrocytes as APC in vivo has not been eliminated, the evidence in the literature indicates that the level of class II expression by astrocytes in vivo may not be significant in comparison with microglia. Inasmuch as multiple neurotransmitters are likely to be operative simultaneously, inhibition of class II in astrocytes in vivo may be achieved by much smaller concentrations of a number of transmitters instead of the rather high dose of glutamate or norepinephrine required for complete inhibition shown in this study.

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